Toxicity Assessment of Phytogenic Microemulsions against the Larvae of *Anopheles stephensi* and *Aedes aegypti*

SYNOPSIS

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INTRODUCTION

Vectors can be defined as the organisms that infect or harm a host by transmitting a disease causing pathogenic entities like virus, bacteria, and protozoa **(Purcell and Almeida, 2005)**. More than 700,000 people die every year due to vector-borne illnesses accounting for more than 17% of all infectious diseases **(WHO, 2020)**. Mosquitoes are contemplated as important and most diverse arthropod-vectors responsible for spreading and transmitting more diseases than any other vector affecting a number of individuals worldwide **(Naseem *et al.,* 2016).** In spite of several years of managing and controlling, mosquito-borne diseases are on the rapid rise affecting every age group leading to considerable proportion of world-wide mortality and morbidity **(Tolle, 2009)**, posing a serious threat to over 40% population of the globe **(Jones *et al.,* 2021).** Mosquitoes primarily belong to *Aedes*, *Anopheles* and *Culex* genera and have been identified as deadly vectors for transmitting diseases such as Yellow fever, Dengue, Zika, Chikungunya, Malaria, Encephalitis, Filariasis, West Nile virus fever etc. **(Ghosh *et al.,* 2012; Murugan *et al.,* 2018)**. Malaria has been attributed to slower economic growth as GDP of malaria suffering nations grew 0.25–1.3% less per capita/year than malaria-free ones **(Action and Investment to defeat Malaria, 2015)**. Approximately, 241 million malarial cases are reported in 2020 and India contributed nearly 83% of all malarial deaths in South-East Asia **(WHO, 2021)**. Dengue, an acute mosquito-borne viral infection, infects 400 million people every year **(Salam *et al.,* 2018)**. Asia is accounting for 75% of the dengue cases, followed by Latin America and Africa **(Wilder-Smith *et al.,* 2019).**

Unregulated and sequential utilization of highly hazardous insecticides like organophosphate, organochlorine and synthetic pyrethroids in various mosquitocidal products, results in multiple insecticide-resistant mosquito vectors as well as detrimental ecological impacts including death of non-targeted organisms **(Pino-Otín *et al.,* 2019)**. In India, mosquitoes have been reported to be insusceptible to Malathion, Dieldrin, DDT, Carbamate and pyrethroid insecticides **(Raghavendra *et al.,* 2011; Karunaratne *et al.,* 2018)**. As a result, safer, environment-friendly, target specific and cost effective alternatives are needed to consistently deliver higher responsiveness to the targets as an acceptable option for management and control of mosquito vectors.

For serving this purpose, the plant-based products are used alternatively and extensively as a source of

phytopesticides over the several decades **(Das *et al.*, 2007; Ponsankar *et al.*, 2020).** Several plants are used for research because of easily available resources and the presence of various phytochemicals like terpenoids, alkaloids, phenolics, catechins and saponins which have insecticidal properties **(Adewole *et al.*, 2013; Danlami *et al.*, 2014; Pavela *et al.*, 2019).** In contrast to other plants species evaluated, *Rumex obtusifolius* or bitter dock, is a perennial invasive plant or weed of grasslands found nearby to meadows and pastures, abandoned fields, field borders, hedgerows and orchards and can remain viable for a long time and thus lead to disturbance or eutrophication in agricultural practices. *Terminalia arjuna*, commonly known as Arjuna, is a medicinally, culturally and economically important plant. It is a typical avenue tree occurs naturally in many cities, is common throughout the India, can be frequently discovered beside waterways and used in multipurpose ways like to treat mild hypertension, Asthma, skin diseases, diabetes, cough, as a cardiovascular drug etc. The plants are known for their hyperglycemic, antiallergic, antioxidant, antimicrobial, antitumoral, antiparasitic, anticarcinogenic, larvicidal, insecticidal and pesticidal properties. The phytocompounds like Hexadecanoic acid, Palmitic acid, Tritetracontane, Arjunic acid, Arjunolic acid, Arjungenin Pyragallol and Supraene belonging to various classes like Phenols, fatty acids, terpenoids, flavonoids are also present which have both insecticidal and pesticidal properties **(Kim *et al.*, 2002; Ginovyan *et al.*, 2021; Dwivedi and Chopra, 2014; Amalraj and Gopi, 2017; Gomathy and Rathinam, 2017)**. So, on the basis of economic importance, essentiality, abundance, availability, and pharmacological history of *Rumex obtusifolius* and *Terminalia arjuna* are the suitable plants species to consider for the various studies.

Although, there are few challenges with the direct application of plant materials such as their low water-solubility which require high amount of solvents to solubilize, less stability and utilization of bulk plant material to get the required amount of active ingredient. Nano-science provides a new approach to manipulate the materials at atomic, molecular, and macromolecular levels, known as "Nanotechnology" via use of nanoparticles, nanofibers, nanoemulsions and nanocapsules **(Hannah and Thompson, 2008; Agrawal and Rathore, 2014).** Nanotechnology involving the strategy of "controlled release of the active components" reduces pesticide intake and related environmental concerns and increase formulation efficacy **(Manjunatha *et al.*, 2016)**. Micro- and Nanoemulsions

(MEs and NEs) are considered as the most versatile nano-structured materials, dimension ranging between 1 to 100 nm including an aqueous phase, an oily phase, a surfactant and co-surfactant as main components **(Abou El-Nour, 2010; Pavoni *et al.*, 2019)**. MEs are optically clear, low viscous and thermodynamically stable nanoformulations **(Sieniawska *et al.*, 2019; El-Sawi *et al.*, 2020)**. Due to their improved bioavailability, absorption behavior, emulsification of weakly water-soluble substances, easy to prepare, long shelf life, considered as ideal for delivery system **(Pavela *et al.*, 2019; Yin *et al.*, 2021; Pavela *et al.*, 2021)**. Hence, MEs are applicable for integration of extracts as well as for both hydrophilic and hydrophobic drugs deliveries to the target organisms **(Asghar *et al.*, 2021)**.

The enzymes mainly esterases (acetylcholine and carboxyl esterase), phosphatases (acid and alkaline phosphatase) and glutathione S-transferase (GST), play an important role in regulation of physiological functions and improve metabolism in insects **(Parthiban *et al.*, 2020; Ali *et al.*, 2021)**. The alterations in physio-chemical processes and the behavior of insects may be linked to effect of pesticides **(Parthiban *et al.*, 2020)**. Synthetic pesticides can have detrimental effects on humans and the environment, also toxic to non-target organisms, which has a negative influence on biodiversity **(Lengai *et al.*, 2020**). Non-target toxicity analysis assesses the detrimental effects of pesticides on non-target organisms **(Benelli *et al.*, 2018).** Additionally, residual toxicity analysis is helpful to assess insecticides with low toxicity and duration of effects on targets as well as non-target organisms **(Roubos *et al.*, 2014).**

Therefore, the present study is intended to assess the larvicidal bioefficacy of *Terminalia arjuna* and *Rumex obtusifolius* extracts and their microemulsions against *Anopheles stephensi* and *Aedes aegypti*. The enzyme activity, in order to analyze the physiochemical changes along with residual and non-target toxicity of the micro-emulsions against *Daphnia* and *Cyclops* will also be studied. Hence, the proposed research work is a step ahead in the development of efficient, cost-effective and eco-friendly larvicidal formulation against *Anopheles stephensi* and *Aedes aegypti*.

REVIEW OF LITERATURE

Mosquitoes cause a wide range of illnesses notably Encephalitis, Malaria, Yellow Fever, Dengue Fever, and Filariasis. Among the multiple mosquito control approaches, the use of larvicides remains

one of the most effective strategies. The synthetic pesticides cause a wide range of natural issues, including the evolution of resilient insect strains, ecological degradation and mammalian toxicity. Therefore, the technocrats diverted their attention towards the efficacy of the phyto-compounds and their role in mosquito control. The literature consulted in this regard is summarized as under:

PHYTO-EXTRACTS AND MOSQUITOES:

Mohan *et al.*, 2005 tested the efficacy of *Solanum xanthocarpum* fruits against *Anopheles stephensi* and *Culex quinquefasciatus* and observed Petroleum ether extract as the potent extract against *Cx. quinquefasciatus* and carbon-tetrachloride extract against *An. stephensi* after 24 hours. **Sharma *et al.* (2006)** evaluated the larvicidal effect of *Artemisia annua, Chenopodium album* and *Sonchus oleraceus* against *Anopheles stephensi* and found Petroleum ether extract of *A. annua* as the most potent extract with lowest LC_{50} value. **Sharma *et al.*, 2009** determined Petroleum ether extract of *Amaranthus oleracea* as an efficacious larvicide against *Anopheles stephensi* after 24 and 48 h of treatment. **Kamaraj *et al.* (2011)** determined larvicidal properties of different solvent extracts of bark and dried leaf of *Chrysanthemum indicum, Tridax procumbens* and *Annona squamosa* against the *Anopheles subpictus* and *Culex tritaeniorhynchus* and observed the highest toxicity in leaf ethyl acetate extract of *C. indicum* in case of *An. subpictus* and leaf methanol extract of *C. indicum* against *An. subpictus* after 24 h of exposure. **Pushpalatha *et al.*, 2012** evaluated the effect of acetone extracts of fruit of *Anamirta cocculus* and leaves of *Sphagneticola trilobata* against *Culex pipiens* and determined that *A. cocculus* has more activity than the leaf extract of *S. trilobata* after 24 hours. **Govindarajan *et al.*, 2012** reported the maximum bioefficacy of methanol extract of *Ervatamia coronaria* and *Caesalpinia pulcherrima* and with LC_{50} values 86.47 and 113.26 ppm against *Anopheles subpictus* and 131.53 and 165.28 ppm *Culex tritaeniorhynchus*, respectively. **Poonia and Kaushik (2013)** assessed the larvicidal effect of Sausage tree (*Kigelia africana*) and Karanj (*Pongamia pinnata*) against the larvae of *Aedes aegypti* and results showed that *P. pinnata* and *K. africana* possess high larvicidal activity. **Pavananundt *et al.*, 2013** tested *Cassia siamea* leaf aqueous extract against the *Aedes aegypti* and reported the toxicity and histopathological effects of the *C. siamea* as a bioinsecticide in the midgut. **Shivakumar *et al.* (2013)** checked the bioefficacy of leaf extracts of *Elaeagnus indica, Memecylon edule, Blepharis maderaspatensis, Phyllanthus wightianus*

and *Maesa indica* against *Aedes aegypti* larvae and observed the maximum larval mortality in acetone extract of *E. indica*. The larvicidal potential of methanolic extract of *Chromolaena odorata* leaves was tested against *Culex quinquefasciatus, Aedes aegypti* and *Anopheles stephensi* and the highest mortality was reported in case of *Cx. quinquefasciatus* **(Sukhthankar *et al.*, 2014)**. **Hemavathy and Anitha (2016)** evaluated the larvicidal bioefficacy of *Vitex negundo* and *Duranta repens* against *Culex quinquefasciatus* and reported that distilled water, methanol and ethanol extracts showed the 100% mortality. **Sumathi and Gaspar, 2016** evaluated the larvicidal and pupicidal effect of acetone extract of *Terminalia arjuna* against the dengue vector, *Aedes aegypti* and found it more effective against 1st instar larvae and pupae at highest dose. **Veni *et al.* (2017)** tested different solvent extracts of *Terminalia chebula* to check the ovicidal and larvicidal potential against *Aedes aegypti*, *Culex quinquefasciatus* and *Anopheles stephensi* and reported the highest larval mortality in the methanol extract of *T. chebula* against the larvae of *A. stephensi*. **Hari and Mathew (2018)** determined the bioefficacy of extracts prepared from leaves of *Tecoma stans*, *Nerium oleander* and *Hyptis suaveolen* against *Aedes aegypti* and *Culex quinquefasciatus* and observed the maximum larvicidal activity in case of PE extract of *L. camara*. **Ravi *et al.*, 2018** tested the larvicidal effect of methanolic extract of *Azolla pinnata* against *Aedes*, reporting the maximum values in its Soxhlet extraction. The larvicidal bioefficacy of aqueous leaf extracts of *Ailanthus excels, Aloe vera, Clerodendrum infortunatum* and *Sesbania grandiflora* on larvae of *Aedes albopictus* was assessed by **Indusree *et al.*, 2019** and highest mortality rate was observed in aqueous leaf extracts of *C. infortunatum*. **Ravindran *et al.* (2020)** determined the chemical composition of *Clitoria ternatea* flowers extract and also checked its larvicidal potential against the larvae of *Aedes albopictus* and *Aedes aegypti* and observed the highest larvicidal activity against *Aedes aegypt*. **Ashok and Babu (2021)** reported the larvicidal effect of weed *Setaria viridis* extracts against *Anopheles stephensi* larvae and stated that extracts of *S. viridis* is more potent in case of *Anopheles stephensi* as compared to *Culex quinquefasciatus.* **Salama *et al.* (2022)** assessed the larvicidal activity of *Rumex vesicarius* against *Aedes aegypti* and found it as a potential tool for controlling dengue vector.

PHYTO-EXTRACTS AND MICROEMULSIONS:

Cespi *et al.*, 2017 tested the cytotoxicity of *Smyrnium olusatrum* essential oil loaded microemulsions on human colon carcinoma cells (HCT116) and results showed that microemulsions were active on HCT116 human colon carcinoma cell lines. **Campana *et al.* (2017)** investigated the antibacterial activity of *Salvia officinalis* and *Cinnamomum cassia* and both essential oils loaded microemulsions and results showed up to 68% of biofilm removal after 90 min of exposure. **Rivera-Rangel *et al.* *in* 2018** formulated silver nanoparticles from Geranium leaves extract loaded microemulsions as results indicated that microemulsion medium controls the size of the silver nanoparticles better. **Pavoni *et al.*, 2019** formulated the microemulsions of *Crithmum maritimum, Trachyspermum ammi* and *Pimpinella anisum* oil and their efficacy was checked as the antibacterial and antifungal agents. **Quintão *et al.* (2019)** formulated MEs loaded with ethanolic extracts of *Brosimum gaudichaudito* and determined their activity for pigmentation and melanocyte migration and it was observed that the microemulsions controlled the permeation of the furanocoumarins through the skin. **Salimi and Shirani, 2021** prepared the microemulsions with the ethanolic and methanolic extracts of *Ziziphus spinachrist* and checked their antifungal activity against *Candida* species. The results indicated that MEs containing *Ziziphus spinachrist* were most inhibitory in nature against *C. albicans*. **Degot *et al.* (2021)** formulated *Curcuma longa* based surfactant-free microemulsion extraction systems (SFME) over the conventional separation methods for the extraction of curcuminoids. The anti-inflammatory activity of *Withania coagulans* extract loaded microemulsion was checked by **Asghar *et al.*** in **2021** and observed that 10% extract loaded MEs showed better anti-inflammatory activity that other formulated MEs.

MICROEMULSIONS AND MOSQUITOES:

Montefuscoli *et al.* (2014) prepared oil in water (o/w) Geranium essential oil (EO) loaded microemulsion and assessed their larvicidal bioefficacy against *Culex pipiens* and result showed 100 % mortality until the 5th day of storage of the potent MEs. **Navayan *et al.*, 2017** formulated *Eucalyptus globulus* essential oil (EO) MEs and tested their repellency against Culicidae larvae and reported the maximum repellency in case of 15% w/w *Eucalyptus globulus* essential oil MEs (170 ± 27 minutes). The neem oil loaded microemulsion with biodiesel waste as co-solvent were prepared and their bioefficacy was checked against *Aedes aegypti* larvae by **Iqbal *et al.* (2018)** and the observed

LC_{50} values were 56.985, 52.604 and 38.575 for neem oil microemulsions. The bioefficacy of MEs loaded with *Pimpinella anisum*, *Trachyspermum ammi* and *Crithmum maritimum* oil was evaluated against the mosquito vector, *Culex quinquefasciatus* and also against non-targets including *Tubifex tubifex*, *Daphnia magna* and *Eisenia fetida* **(Pavela *et al.*, 2019)** and inferred that both antifungal as well as antibacterial activities were enhanced when essential oils were encapsulated in MEs. The larvicidal efficacy of *Carlina acaulis* root essential oil loaded MEs was determined against the larvae of *Culex quinquefasciatus* **(Pavela *et al.*, 2021)** and maximum larvicidal activity was observed by the 0.5% ME with LC_{50} value of 579.1 µL/L. **Soroh *et al.*, 2021** determined the mosquito repellent activity of Lemon and Rosemary EO-loaded microemulsions against *Aedes aegypti* and maximum mean repellency observed was 72.9 ± 13.9%.

PHYTO-EXTRACTS AND ENZYMATIC ACTIVITIES:

Zahran *et al.* (2017) tested the inhibitory effect of *Schinus terebinthifolius*, *Artemisia monosperma* and *Origanum vulgare* EOs on Acetylcholinesterase and adenosine triphosphates activities in larvae and adults of *Culex pipiens*. Among all the EO, *O. vulgare* oil (IC_{50} = 12.75 mg/l) was observed to be most potent enzyme inhibitor. **Thanigaivel *et al.*, 2017** studied the effect of leaf extracts of *Justicia adhatoda* on the activity of α-carboxyl esterase and β-carboxyl esterase enzymes in *Aedes aegypti*. It was seen that activity of GST and CYP450 activity were increased while the activity of α- and β-carboxylesterase and superoxide dismutase were reduced. **Pavunraj *et al.*, 2017** checked the inhibitory effect of *Catharanthus roseus* and *Acalypha fruticosa* extracts on enzymes including α carboxyl esterases and β carboxyl esterase in *Culex quinquefasciatus* and results revealed that α- and β- carboxylesterase activities increased in *C. quinquefasciatus* to various solvent extracts of *A. fruticosa* and *C. roseus.* The neurological and metabolic changes induced post treatment of ar-turmerone extracted from *Curcuma longa* in *Culex pipiens* larvae were determined by **Liu *et al.*, 2020** by checking the activities of the acetylcholinesterase (AChE), glutathione-S-transferase (GST), Cytochrome P450 monooxidases (P450) and carboxylesterase (CarE) and it was observed that the activities of GST, CarE and P450 were increased but there was no significant effect on the level of AChE activity**. Parthiban *et al.*, 2020** determined the enzyme inhibitory effects of *Annona muricata* extracts on α-carboxyl esterase, β-carboxyl esterase and acetylcholinesterase (AChE) in *Anopheles*

stephensi, *Aedes aegypti* and *Culex quinquefasciatus* and this study suggested the decrease in the activity of α-and β-carboxylesterase and acetylcholinesterase (AChE). **Prakash *et al.* (2021)** assessed the changes in the level of *α*-carboxyl esterase, *β*-carboxyl esterase, glutathione S-transferase (GST) and cytochrome P450 (CYP450) in *Culex quinquefasciatus*, *Aedes aegypti* and *Anopheles stephensi* induced due to the impact of root Extract of *Decalepis hamiltonii.*

PHYTO-EXTRACTS AND NON TARGETS:

Pavela and Govindarajan, 2017 tested the larvicidal effect of *Zanthoxylum monophyllum* leaf essential oil against non-target, *Gambusia affinis* and found it safer against the non-target fish. **Govindarajan *et al.*, 2016** assessed the toxicity of Ag nanoparticles synthesized using *Nicandra physalodes* aqueous extract against non-target water bugs and observed toxicity was negligible with LC_{50} values ranging from 1,032 to 19,076 µg/mL. The toxicity of *Aglaia elaeagnoidea* or Priyangu-synthesized Ag nanoparticles checked against non-targets including larvivorous fishes, backswimmers, and water bugs by **Benelli *et al.* (2018)** and the nanoparticles exhibited non-toxicity with LC_{50} values ranged from 1247-37,254.45 µg/mL. **Kamaraj *et al.* (2018)** reported effect of methanol extract of *Actiniopteris radiata* leaves on embryos of *Danio rerio* (Zebra fish) and *Artemia nauplii* (micro- crustacean and reported insignificant effect of extracts against the non-targets. **Minal and Prakash (2020)** reported the toxicity of *Citrus limon* mediated Au–Pd bimetallic nanoparticles against nymphs of Dragonfly and Damselfly and observed no changes in their predation efficiency. **Esan *et al.*, 2021** evaluated the effect of *Alstonia venenata* AgNPs on non-target fish and insects and found them safer against the selected non-targets. **Sánchez-Gómez *et al.* (2022)** assessed the toxicity of *Pimpinella anisum* and *Trachyspermum ammi* EO-loaded zein nanocapsules against aquatic (*Daphnia magna*) and terrestrial (*Eisenia fetida*) invertebrates and reported the negligible or limited lethal effect of prepared formulation against the non-targets.

According to the above described literature, it has been concluded that the plant based products may be used as efficacious and eco-friendly alternatives to the synthetic insecticides. However, their nanoformulations mainly microemulsions have received comparatively limited focus in mosquito control. Therefore, the proposed study has been designed to evaluate the phytoextract mediated larvicidal efficacy as well as their microemulsions loaded with the extracts on mosquito vectors,

Anopheles stephensi and *Aedes aegypti* along with the biochemical study to determine the possible mechanism of action that causes the enzymatic or physiological alterations if any in selected mosquito species. Further, the above phytogenic microemulsions will also be evaluated against aquatic non-target organisms to check their eco-friendly nature to the environment.

OBJECTIVES

The main objectives of the proposed study is to control the mosquito-borne diseases through the management of mosquito larvae using phytogenic micro-emulsions which will be achieved through-

1. Extraction, characterization and bioefficacy of the selected phytoextracts against *Anopheles stephensi* and *Aedes aegypti* larvae.
2. Preparation and characterization of phytogenic microemulsion using the most effective extract and their larvicidal efficacy against the target organisms.
3. Analysis of residual toxicity against the target organism to assess the persistency of the prepared phytogenic microemulsions in the environment.
4. Assessment of Aquatic non-targets toxicity of the prepared phytogenic microemulsions to evaluate their eco-friendly nature.
5. Assessment of the microemulsion on the enzymes of target organisms to understand their possible mode of action.

MATERIALS AND METHODS

(I) MATERIALS:

- **Target organisms:** The larvae of *Anopheles stephensi* and *Aedes aegypti*.

They are restricted to aquatic habitats and are unable to escape control measures whereas adults are highly mobile flying insects that can detect and escape many intervention measures.

A. *Anopheles stephensi*

Anopheles stephensi belongs to class-Insecta, order-Diptera and family-Culicidae, is the most common malarial vector in India, contributing about 12% of total malaria cases **(Tyagi *et al.*, 2017)** and found mainly in urban areas, called as the urban malaria mosquito. *Plasmodium falciparum* and *Plasmodium vivax* are the most common malarial parasites which are known to be transmitted by *An. stephensi* **(Balkew *et al.*, 2020).**

Habitat - *An. stephensi* tends to adapt well to the local environment, breeds in artificial as well as natural habitats like garden reservoirs, household water storage containers, clean and fresh water reservoirs like ponds, lakes, swamps, slow-running streams, marshes etc.

Identifying features - Predominantly dark legs with pale and dark scales, palmate hair on abdominal segments, wings with lightly spotted veins, maxillary palpus with pale bands. Proboscis and body align in same straight line at 45^0 from surface while biting and active between sunset and sunrise. Larvae float horizontally or parallel to the surface of water and rudimentary breathing tube is present.

B. *Aedes aegypti*

Aedes aegypti belongs to Class-Insecta, Order-Diptera and Family-Culicidae. Out of 950 species of *Aedes*, *Ae. aegypti* is known to transmit Yellow fever, Dengue, Dengue hemorrhagic fever and other viral infections.

Habitat - Common habitats include clean water sources generally like water storage tanks, jars, drums, tyres, tin cans, bottles and plant pots, roof gutters, bamboo stumps placed outsides or insides.

- **Identifying features** - Abdomen with white, pale or yellow scaling, white spotted legs, maxillary palps are shorter than the proboscis, white scales on the top of the thorax forming the shape of a violin or lyre, wings with black and white bands, proboscis and body lies at angle to one another while biting, active in the morning or evening. Larvae rest at an angle to the water surface, white in color except head and siphon which are black in color, short breathing tube with one pair of hair tufts.
- **Plants Selected:**

1. *Rumex obtusifolius* (family- Polygonaceae)

It is commonly called as the Broad-leaved dock, is a herbaceous, broadleaved, perennial weed of economic importance, native to all the temperate continents including Asia and mainly Europe and grows up to generally 40-150 cm. As per the **UK Weed Act, 1959**, it is listed as "injurious weed". It is found nearby or at sides of roads, rivers, lakes, abandoned fields, ditch banks, streams, hedgerows, ditches, meadows or at terrestrial and wetlands. Basal leaves are smooth, broad, ovate, and usually red-veined with heart-shaped base. It acts as curative for cancer, malignancies, sores, blisters and burns and also used as tonic, laxative, depurative and astringent **(Harshaw *et al.*, 2010)**. **Feduraev *et***

***al.*, 2019** showed the presence of catechins, phenolic compounds, proanthocyanidins (condensed tannins) and it also exhibits antibacterial properties **(Harshaw *et al.*, 2010; Purohit *et al.*, 2020)**, antioxidant capacity **(Nazari *et al.*, 2017)**, hyperglycemic, pesticidal and larvicidal properties **(Kim *et al.*, 2002; Ginovyan *et al.*, 2021)**. On the basis of all these biological properties and economic importance, this plant is considered for present study.

2. *Terminalia arjuna* (family- Combretaceae)

Terminalia arjuna, commonly known as Arjun, Partha, Sadad, Sajada, Matti, Koha and Kahu, is an evergreen tree grows about 20–25 meters tall. It has thick, grey to pinkish green bark that is smooth and thin. Leaves are usually sub-opposite, 10–15 cm long and 4–7 cm wide, with a rounded or heart-shaped base and reticulate veins. Fruit is a fibrous-woody drupe, about 2.5–5 cm in size. It is dark brown when mature and has five hard, projecting, veined wings. Flowers are small and white and Flowering time is from April–July in Indian conditions. It is used for anginal pain, hypertension, congestive heart failure, and dyslipidemia **(Dwivedi and Chopra, 2014).** Arjuna possessed antimicrobial **(Chaudhari and Mengi, 2006)**, antioxidant, hypotensive, anti-atherogenic, anti-inflammatory, anti-carcinogenic, anti-mutagenic, gastro-productive **(Amalraj and Gopi, 2017)** and larvicidal effect **(Gopinath *et al.*, 2013; Gomathy and Rathinam, 2017).**

(II) METHODS

- **Rearing of mosquitoes:**

Larvae will be collected from the natural oviposition areas of Dayalbagh, Agra and maintained at 27±2 ^{o}C and 70–80% relative humidity under a photoperiod of 14:10 hours (light/dark). Pupae will be raised in water and feed on mixture of finely ground dog biscuits and yeast extract and then, they will be transferred from the trays to a container of tap water before being placed in screened cages 23×23×32 cm in diameter for adult emergence. Further, the rearing of adults will be carried out in glass cages 30×30×30 cm in size and will be provided with freshly soaked deseeded raisins as primary diet. Later, female mosquitoes will be allowed for blood feed on albino rats on the second day of emergence after every third day for the egg maturation. The eggs will be gathered in container covered with Whatman filter paper and eggs will be then transferred on dechlorinated water-filled trays to hatch at same

conditions. The 3rd instar larvae will be taken for the bioassay **(Batabyal *et al.*, 2009; Bhan *et al.*, 2015).**

- **Collection of plant materials and preparation of extracts:**

Leaves of *Rumex obtusifolius* and fruits (Drupes) of *Terminalia arjuna* will be collected from the different places of Agra throughout the time they will be available and washed thoroughly with tap water to get rid of unwanted impurities and then dried at room temperature in the shade. The dried leaves will be crushed and grind manually to make a fine powder and drupes will be crushed in mortar- pestle to obtain a powder and then stored in air tight containers. The powdered plant materials will be weighed and subjected to soxhlet's extraction apparatus using solvents of different polarities (petroleum ether, chloroform, methanol subsequently and with water) for 72 hours for each extract. The solvents will be removed using rotary vacuum evaporator to prepare crude extracts. The crude extracts will be weighed and stored in refrigerator below 4° C for further use **(Mohan *et al.*, 2005; Maurya *et al.*, 2009).**

- **Larvicidal bioefficacy:**

The crude extracts will be diluted in ethanol or acetone to prepare stock solutions of the desired strength and different ranges of concentrations will be prepared by diluting the stock solutions in beaker (250 ml) containing 199 ml water and 1ml of desired concentration of stock solution. The larvicidal bioassay will be carried out according to the standard WHO protocol. Twenty, 3rd instar laboratory acclimatized larvae of *Anopheles stephensi* and *Aedes aegypti* will be exposed independently. All experiments will be conducted in triplicates along with control **(WHO, 2005)**.

- **Phytochemical analysis of the Extract:**

To check the presence of bioactive compounds in the most potent extract, the phytochemical analysis will be done. Various tests will be conducted to check the presence of phytosterols, tannins, flavonoids, saponins, alkaloids, glycoside, triterpenoids, polyphenols and proteins **(Shaikh and Patil, 2020)**.

- **Formulation of microemulsions:**

The microemulsions will be prepared using the most effective phytoextract, emulsifiers (Tween 80 as surfactant and Ethanol as co-surfactant) and water. Surfactant and co-surfactant will be mixed in

required ratios and vortexed for 5 min to obtain the surfactant/co-surfactant mixture (SCmix). The extract will be dissolved in SCmix in different ratios to obtain different test concentrations using the magnetic stirrer (800 rpm) for 30 min at room temperature and then water will be added drop-wise manner and stirred continuously till the solution become homogenous, clear and transparent **(El-Sawi *et al.*, 2020; Souza *et al.*, 2021; Yin *et al.*, 2021)**.

- **Bioefficacy:**

The above-mentioned microemulsion will be used for bioassays against the target organisms in accordance with the standard WHO protocol **(WHO, 2005)** mentioned above.

- **Risk assessment of microemulsions against non- target organisms:**

Prepared microemulsions will also be tested against the aquatic non-target organisms including *Daphnia* and *Cyclops* to observe the toxicological effects if any **(Sanchez Gomez *et al.*, 2022)**.

- **Data analysis:**

The larval mortality of larvae will be observed after 24 and 48 hours of exposure. The larval mortality in the control if ranges between 5 to 20%, then the mortalities of the treated groups will be corrected using Abbott's formula **(Abbott, 1925)** to remove the factors other than the extract, if mortality exceeds 20% then the experiment will be discarded and repeated again. Lethal concentrations (LC_{50} and LC_{90}) values as well as other statistical attributes will also be examined using the Probit analysis **(Finney, 1971)**.

- **Enzyme Assay:**

To study the impact of the most effective phytogenic microemulsions on the enzymatic activities, the treated and control larvae will be washed independently and clean with double distilled water and adhering water will be wiped out from the surface using tissue paper. The larvae will be homogenized in eppendorf tubes with ice-cold sodium phosphate buffer (20 mM, pH 7.0) using a handy homogenizer **(Prakash *et al.*, 2021)** and further subjected for centrifugation for 20 min at 8000× g at 4 ˚C and the supernatants will be separated and kept at 4 ˚C to study Acetylcholinesterase, Carboxyl esterase, Alkaline phosphatase and Glutathione-S-transferase activities **(Karthi *et al.*, 2020; Fouad *et al.*, 2018)**.

1. Glutathione-S-Transferase Activity -

The activity of Glutathione S-transferase enzyme will be studied by the method of **Devonshire *et al.*, 1992** where 20 µl of homogenates will be mixed to 200 µl of reduced glutathione (10 mM) and CDNB (63 mM) mixture solution and then incubated at room temperature (RT) for 5 min. Sample will be run through spectrophotometer and optical density will be noted at 340 nm against a suitable blank **(Parthiban *et al.*, 2020).**

2. Carboxyl esterase Activity -

The levels of Carboxyl esterase in the larval homogenates will be measured by the methodology adapted of **Van Asperen, 1964**. 20 µl of larval homogenates will be added with 1 mL of sodium phosphate buffer (20 mM, pH 7.0) contained α- or β-naphthyl acetate (250 µM) and incubated at RT for 30 min. Later, 400 µl of fast blue B (0.3%) with sodium dodecyl sulphonate or SDS (3.3%) will be added to this mixture to stop the enzymatic reaction and then allowed for color change at RT for 15 min. The optical density of each reaction mixture will be observed at 430, 588 nm for α- or β-carboxylesterase respectively using spectrophotometer and standard curve will be plotted against α- or β-naphthol as standard to calculate the activity of α- or β-carboxyl esterase **(Parthiban *et al.*, 2020).**

3. Acetylcholinesterase Activity -

To check the Acetylcholinesterase activity in the larval homogenate, the method of **Ellman *et al.*, 1961** will be adapted. 100 µl of enzyme solution from whole body homogenates will be added to 50 µl of acetylthiocholine iodide as a substrate and 1 ml of sodium phosphate buffer (20 mM, pH 7.0) and then the mixture will be incubated at 25ºC for 5 minutes. The reaction will then be stopped by adding 400 µl of freshly made 0.3 percent fast blue B salt in 3.3% SDS. The optical density of the sample will be observed at 405 nm using a spectrophotometer **(Ali *et al.*, 2021).**

4. Alkaline Phosphatase Activity -

The alkaline phosphatases (ALP) activity in the larval homogenates will be measured by the method of **Asakura, 1978**. The homogenate will be mixed with Tris-HCl buffer (pH 8.0) and p-nitro phenyl phosphate and incubation for 15 minutes at 37°C in water bath, the enzymatic reaction will be stopped by adding NaOH solution and then centrifuged at 4000 rpm for 5 minutes and the supernatant will be noted at 440 nm against blank **(Ga'al *et al.*, 2018).**

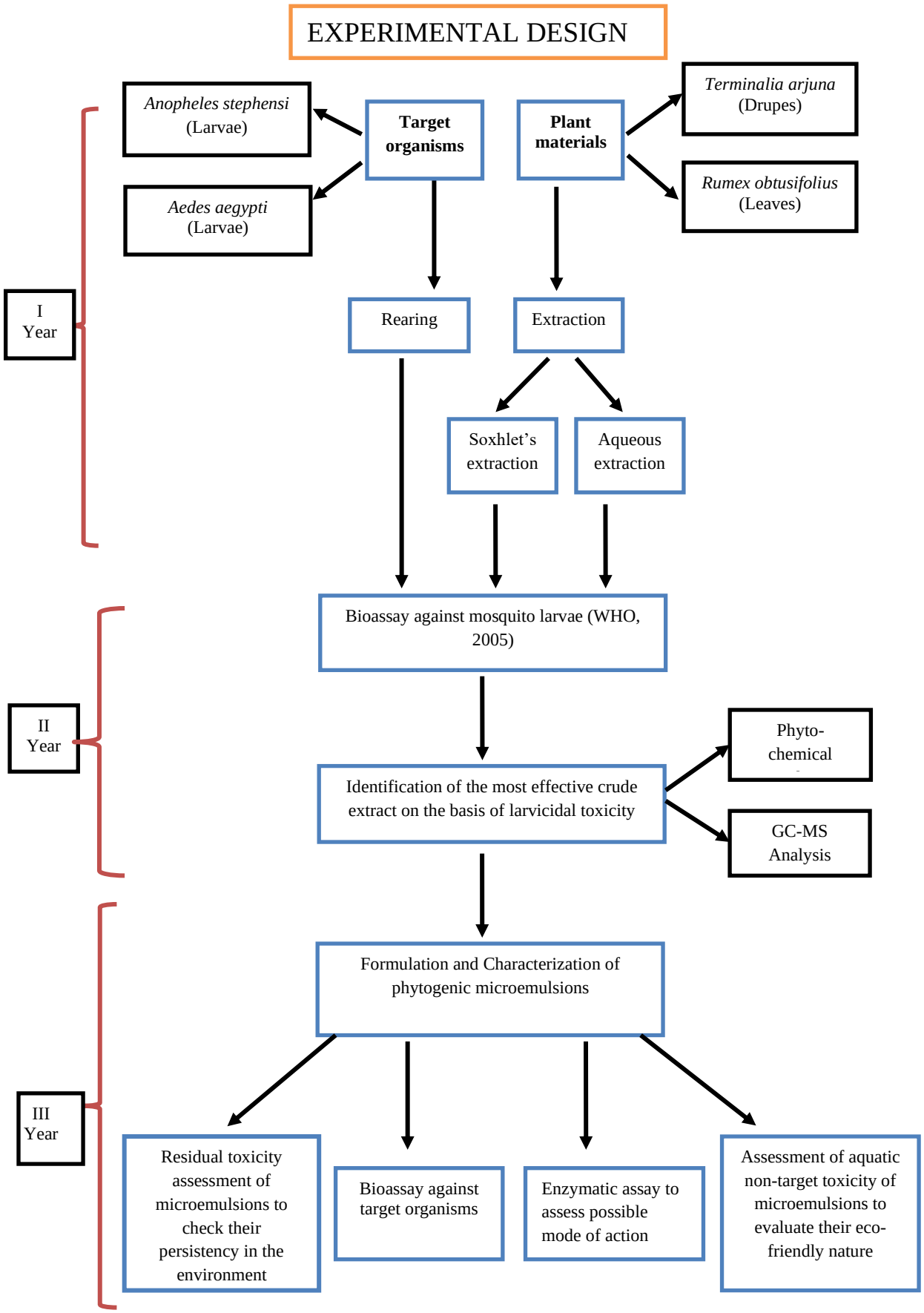
EXPERIMENTAL DESIGN
Anopheles stephensi (Larvae)
Aedes aegypti (Larvae)
Target organisms
Plant materials
Terminalia arjuna (Drupes)
Rumex obtusifolius (Leaves)
I Year
Rearing
Extraction
Soxhlet's extraction
Aqueous extraction
Bioassay against mosquito larvae (WHO, 2005)
II Year
Identification of the most effective crude extract on the basis of larvicidal toxicity
Phyto-chemical
GC-MS Analysis
Formulation and Characterization of phytogenic microemulsions
III Year
Residual toxicity assessment of microemulsions to check their persistency in the environment
Bioassay against target organisms
Enzymatic assay to assess possible mode of action
Assessment of aquatic non-target toxicity of microemulsions to evaluate their eco-friendly nature

Fig. 1: Plan of Work

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